Technical notes

There's something about glutathione

Introduction

In 2015 the International Organisation of Vine and Wine (OIV) passed two resolutions recommending that reduced glutathione (GSH) be allowed to be used as an additive to grape juice or wine (OIV-OENO 445 and OIV-OENO 446). However, pure GSH is not currently a permitted additive and would require inclusion in the food standards codes of individual wine-producing countries before it could be used. Nonetheless, GSH is a component of many fermentation aids, is produced naturally by yeast during fermentation, and might one day become a permitted additive. This article provides a description of GSH, how its use is being considered in the context of winemaking, and reports on some work undertaken as part of a collaborative project between the AWRI and the University of Adelaide exploring its potential as a winemaking additive.

What is glutathione?

Glutathione is a low molecular weight metabolite present in organisms as diverse as bacteria, fungi, plants and animals (Kosower & Kosower 1978). It consists of three amino acids – glycine, cysteine and glutamate – and, within a cell, exists in two forms, predominantly as a reduced species (GSH) and as a lower proportion in an oxidised form (GSSG). For simplicity, in this article the word glutathione is used to represent total (GSH/GSSG). The major role of GSH within a cell is to neutralise and remove reactive oxygen species formed through respiration (i.e. it acts as an antioxidant).

What does glutathione have to do with winemaking?

GSH is present in both grapes and yeast (Kritzinger et al. 2013a). Its concentration in grape juice is dependent on the conditions under which grape processing is undertaken. During the harvesting and processing phases of winemaking, oxygen has the opportunity to react with oxidase enzymes and phenolic compounds in the berry. The reaction results in the formation of reactive oxygen species and quinones which then interact with GSH to stimulate its loss from juice or must (Singleton et al. 1985). High concentrations of SO_2 and/or protection from oxygen are required to prevent this type of reaction. Therefore, the primary factor influencing the concentration of GSH in must is the exposure to oxygen of grape berries during berry rupture (du Toit et al. 2007, Motta et al. 2014). Protection from air and oxidation, using an inert press for example, will generate juice with higher GSH and polyphenol concentrations. GSH concentrations in excess of 50 mg/L have been reported in juice following inert pressing (Pons et al. 2015), with some proportion of that still present at the conclusion of fermentation.

Aside from grapes, other potential sources of GSH in wine include yeast-derived products, some of which are marketed as GSH-enriched inactive dried yeast preparations. However, compared to grape processing interventions that can preserve GSH in juice or must, the GSH contribution of these additives is small, increasing GSH concentrations by between 1 and 3.5 mg/L when used at recommended addition rates (Kritzinger et al. 2013b, Andújar-Ortiz et al. 2014, Rodriguez-Bencomo et al. 2014). Yeasts can also contribute GSH to ferments and finished wines through synthesis and export of the molecule during fermentation. Glutathione is the main sulfur compound in yeast and is produced and metabolised in ways that depend on yeast exposure to a variety of stresses (Penninckx 2000).

In work undertaken by the project team, the evolution of glutathione was monitored in pilot-scale fermentations (50 L) of Chardonnay and Riesling, which were then bottled at two SO_2 concentrations (Figure 1). Both juices were prepared using standard aerobic pressing methods. At the start of fermentation glutathione was barely detectable. It accumulated continuously, reaching approximately 14 and 9 mg/L in the Chardonnay and Riesling wines respectively, by ferment completion. The Chardonnay fermentations were sluggish, with remedial action undertaken after 20 days, but they eventually completed after 44 days. During cold settling of the finished wines, glutathione concentrations continued to decrease moderately in Chardonnay and increased in Riesling, reaching respective concentrations of 8 and 12 mg/L before bottling. Cellaring of the wines for a further three months saw GSH concentrations decline in both wines, with the higher concentrations of SO_2 associated with accelerated decrease of GSH in the Chardonnay wine. Both the overall concentrations of GSH observed in these wines and the SO_2-associated decrease in GSH are consistent with the observations of others (Panero et al. 2015, Pons et al. 2015).

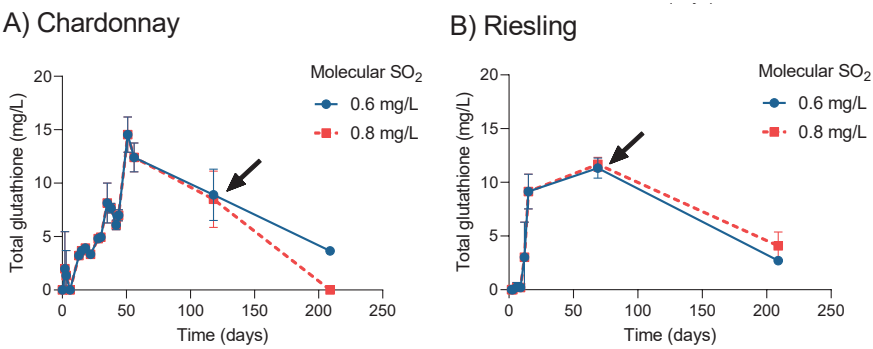

Figure 1. Evolution of glutathione concentration during fermentation (50 L) of Chardonnay (A) and Riesling (B) from pressing of juice to wine bottling and during bottle ageing. Both wines were bottled at two SO_2 concentrations, 0.6 and 0.8 mg/L molecular SO_2. Arrows indicate pre-bottling SO_2 additions, after which wines were bottled and cellared. Error bars show standard deviation (n=3).

In summary, glutathione is a normal constituent of grapes and yeast and, depending on how the wine is made, will contribute in different ways to the winemaking process. If the juice is produced oxidatively then little GSH will make it to bottle. However, if the wine is made reductively, concentrations of GSH sufficient to participate in post-fermentation processes can be present in the finished wine. Substantial research effort has been expended in order to understand what those processes are, and how any interactions of GSH will affect them.

Effects of glutathione on wine

The oxidative processes that began during grape processing continue once wine is in bottle, and the steps taken during bottling become critical in controlling their progression. The detrimental effects of oxidation, such as the browning of white wine or development of undesirable sensory characteristics, cannot be rectified once wine is in the bottle and therefore the impact is much greater. These effects may be exacerbated if wines are made inertly, preserving phenolic compounds that are then vulnerable to subsequent oxidation. Protection of wines from oxidation is a function that has traditionally been performed by SO_2. However, some wine producers in recent years have sought to minimise concentrations of SO_2 in wine or eliminate its use altogether. In the absence of an effective antioxidant, the shelf life of wine is significantly shortened. GSH is a reducing agent and is capable of preserving volatile sulfur compounds (e.g. varietal thiols) during wine ageing (Nikolantonaki et al. 2014). For these reasons, GSH has been investigated as an alternative antioxidant for wine, and for its potential to act synergistically with SO_2 such that the concentration of SO_2 could be decreased, or even eliminated altogether (Badea & Antoce 2015, Comuzzo et al. 2015).

Panero et al. (2015) investigated the effect of GSH addition on the shelf-life of bottled wines, reporting that post-ferment GSH addition did not help preserve the concentration of SO_2 over time, SO_2 did not preserve GSH concentrations over time, and GSH addition offered no protective effect against white wine browning. The only observed interaction with SO_2 was an accelerated consumption of O_2 in bottle when both GSH and SO_2 were present. Earlier work by Ugliano et al. (2011) showed that post-ferment GSH addition could suppress the loss of varietal thiols such as 3-mercaptohexanol (3-MH) at the expense of higher concentrations of H_2S. For both of these compounds, there were complex interactions with wine copper concentrations and oxygen ingress through the closure. Thus, despite promising theoretical work indicating potentially useful post-ferment applications for GSH, these two studies point toward limited practical outcomes and added risk with potential for in-bottle evolution of unwanted low molecular-weight sulfur compounds.

Some questions remain about the potential for GSH to influence wine style when added prior to, or during fermentation. GSH in grape juice can interact with phenolic compounds

to form grape reaction product, may reverse the oxidation of quinones, and can also form conjugates with grape-derived compounds to generate flavour precursors that can be liberated by yeast during winemaking. The degree to which these reactions occur is governed by oxygen concentration, but the half-life of oxygen is short in freshly prepared juice. Not only does GSH react with the many phenolic compounds present, but it is also a sought-after nutrient for many of the organisms that are present in must. Many of these same organisms, including wine yeasts, also have the capacity to assimilate free GSH (Marsit et al. 2015, Margalef-Català et al. 2016).

With these previous observations in mind, the impact of adding GSH to juice or during the early stages of an active ferment was investigated. This work aimed to understand whether the effects of juice or early ferment additions are similar or whether the early addition is consumed by oxidative reactions and the slightly later addition metabolised. A further question to be investigated was whether the trajectory of wines that had received GSH during fermentation, and where residual GSH was still high at bottling, would be similar to wines that had received GSH only at bottling, as described by Panero et al. (2015).

Exploring the effects of GSH addition prior to or during fermentation

As shown earlier, GSH is present in a wine ferment, whether it is added or not. But what happens when grape-and yeast-derived GSH is augmented with addition of exogenous GSH? This was explored by the addition of excess GSH (250 mg/L) to Chardonnay and Riesling pilot-scale ferments (50 L), both pre- and post-inoculation. The concentrations of oxidised and reduced glutathione were monitored throughout the production of these wines, including during 18 months of cellaring in-bottle (Figure 2).

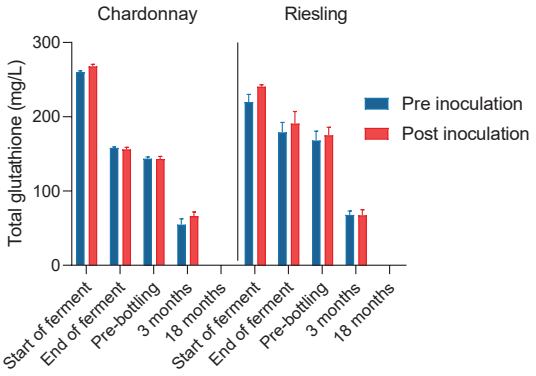

Figure 2. Evolution of glutathione concentrations during and after fermentation in Chardonnay and Riesling wines to which 250 mg/L GSH was added 24 hours before (blue) or 24 hours after (red) inoculation. All treatments were conducted on a 30 L scale in triplicate. Error bars show standard deviation (n=3).

On average, 100 mg/L and 45 mg/L of glutathione was consumed during the course of the Chardonnay and Riesling fermentations, respectively. It made no difference whether the GSH was added prior to or following yeast inoculation. Two-fold higher concentrations of oxidised glutathione (GSSG) were present in ferments when GSH was added prior to inoculation. However, GSSG represented only 5–10% of the total glutathione concentration in those ferments, with the vast majority remaining in the reduced form. Very little glutathione was lost between completion of fermentation and bottling. After three months of cellaring, only 20% (50 mg/L) of the original addition remained, and by 18 months no glutathione was detectable either in reduced or oxidised form.

Extensive analysis of yeast-derived volatiles revealed no substantial changes in the concentrations of the vast majority of odour active volatile compounds. The only exceptions to this were the sulfur compounds 3-mercaptohexanol (3-MH) and hydrogen sulfide (H_2S). Post-inoculation addition of GSH was associated with a 1.5 to 2-fold increase in 3-MH concentrations after three months in bottle (data not shown) and a doubling of H_2S concentrations in Chardonnay was seen following both pre-inoculation and post-inoculation additions (Figure 3). By 18 months the H_2S concentration became excessive, reaching more than 100 µg/L. The timing of GSH addition, either pre- or post-inoculation, did not affect H_2S concentration, but lower concentrations of H_2S were evident in wines with higher concentrations of SO_2 after cellaring for 18 and 22 months. Similar patterns of H_2S development were also observed in Riesling wines, but with 5-fold lower absolute H_2S concentrations.

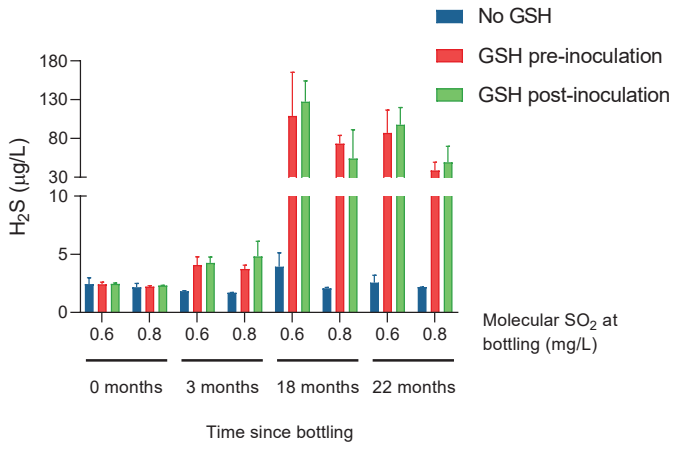

Figure 3. Evolution of hydrogen sulfide concentrations in Chardonnay wines made following addition of GSH pre-inoculation (red bars), post-inoculation (green bars) and without GSH addition (blue bars). The odour threshold for hydrogen sulfide is 1.1 – 1.6 µg/L, as indicated by the dotted line. Error bars show standard deviation (n=3).

What is the effect of juice nitrogen availability on post-ferment glutathione and H_2S concentration?

The OIV resolution on GSH includes a statement that GSH additions should only be made to grape juice when sufficient assimilable nitrogen levels are available to avoid metabolism of GSH by yeast. The appropriate assimilable nitrogen level for this to occur is, however, not specified.

The effect of varying yeast assimilable nitrogen (YAN) concentrations on loss of glutathione during fermentation was evaluated at laboratory scale in defined medium. Figure 4A shows that when GSH was added prior to fermentation, post-fermentation glutathione concentrations were lower than initial glutathione concentrations at all three YAN concentrations – that is, in all cases where GSH was added, its concentration decreased during fermentation. The post-ferment concentration of glutathione was, however, significantly higher at the highest YAN concentration (430 mg/L). The loss of glutathione at all YAN concentrations demonstrates that there is no nitrogen concentration that can completely suppress glutathione loss during fermentation. Whether this glutathione loss is the result of metabolism by yeast remains to be determined. However, fermentative activity was not stimulated by GSH addition at low YAN, indicating that it was not being used as a substitute nitrogen source.

Figure 4B shows the suppression of post-ferment glutathione-related H_2S concentration by nitrogen at YAN concentrations ≥ 300 mg/L. At the lower juice YAN of 150 mg/L, higher GSH concentrations (100 mg/L and 250 mg/L) stimulated higher post-ferment H_2S concentrations in the finished wine. In contrast, at the very lowest GSH concentrations,

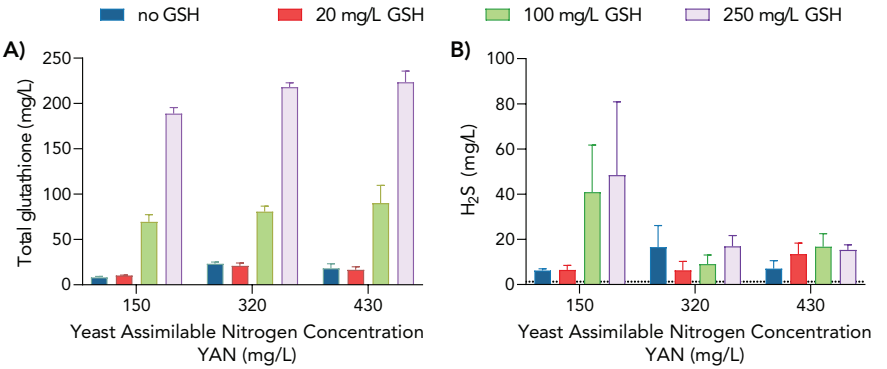

Figure 4. Effect of nitrogen and post-inoculation GSH addition on post-ferment glutathione (A) and hydrogen sulfide (B) concentrations. In plot A the dotted lines indicate glutathione concentrations of the juice prior to fermentation. In plot B the dotted line represents the odour threshold of hydrogen sulfide (1.1 – 1.6 µg/L). Error bars show standard deviation (n=3).

such as those indicated in the OIV resolutions (20 mg/L), there was no difference between it and the control (no addition).

Summary and take-home points

There is something about glutathione; its chemistry and biology is interesting and complex. It is as the centre of so much that is critical to winemaking, from yeast performance to the evolution of key aroma compounds. The idea that there might be an alternative to SO_2 to protect wine against oxidation is a tantalising one. The work conducted here and elsewhere, however, suggests that this will not be the case. There appears to be little protection afforded by GSH at the lower concentrations recommended by the OIV in the two resolutions relating to its use. GSH has not been shown to limit browning in bottle and does not act synergistically with SO_2 such that the lower concentrations of both might be used. At higher GSH concentrations there are risks of undesirable sulfur compounds developing in bottle and these risks do not appear to be easily mitigated through higher nitrogen supplementation. Considering the data presented here and elsewhere, it seems unlikely that an argument for GSH to be added to the relevant food standards codes as a wine additive will be made any time soon.

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Simon A. Schmidt, Research Manager, AWRI, *simon.schmidt@awri.com.au*
Marlize Z. Bekker, Senior Research Scientist, AWRI
Ross Sanders, Research Assistant, University of Adelaide
Kathleen M. Cuijvers, Technical Officer, AWRI
Allie C. Kulcsar, Scientist, AWRI
Dimitra L. Capone, ARC Research Associate, University of Adelaide
Carolyn Puglisi, Research Chemist, University of Adelaide
David W. Jeffery, Associate Professor in Wine Science, University of Adelaide

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